A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF PLASMA PROTEINS.

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In connection with the study of a certain blood reaction in cases of kala-azar (1), we had occasion to determine the different plasma proteins. The refractometric method of Robertson (2) or the recent method of Cullen and Van Slyke (3) based on nitrogen determinations would have served our purpose; but the lack of certain facilities required in these methods compelled us to devise some simpler technique adapted to the equipment at our disposal. As a result of our endeavor we have developed a new colorimetric method which is much simpler than and, we believe, fully as accurate as, any of the other methods.

There are two steps in the determination of the plasma proteins; *viz.*, (*a*) their separation from each other, and (*b*) their quantitative estimation. In the first step we have followed, in the main, the procedure of Cullen and Van Slyke. The isolation of the fibrin by recalcification of the oxalated plasma under the conditions prescribed by these authors is very satisfactory, and we have further simplified the process by whipping the fibrin out of the jelly, thus obviating the necessity of washing. For the precipitation of the globulin they used ammonium sulfate at half saturation. In our study we have found that saturation with magnesium sulfate gives the same results as half saturation with ammonium sulfate, and either process can be used in the new colorimetric method. In the application of this method to clinical cases, however, we have used the ammonium sulfate process which is somewhat more convenient. The magnesium sulfate process was used only when parallel determinations by the Kjeldahl method were desired.

In the second step we make use of the color reaction (4) of proteins with phospho-18-molybdictungstic acid (phenol reagent). Proteins in solution react with this reagent, due largely to the tyrosine which they contain. We have made no experiment to determine what the color produced by the proteins quantitatively represents. But since this chromogenic value is a constant for any given protein, the intensity of the color produced under definite conditions can be used as a measure of the amount of the same protein.

Solutions of pure serum globulin or albumin can, of course, be used for the standard. But as these are laborious to prepare and difficult to keep, we have used exclusively a solution of tyrosine in our work. A convenient standard is made by dissolving 50 mg. of tyrosine in 250 cc. of 0.1 N HCl. We have observed no change in this solution in the course of 6 months and it may keep much longer. Under the conditions described below we have found for human plasma that 1 mg. of tyrosine[1] = 16.4 mg. of fibrin (N × 6.25), 25.2 mg. of globulin (N × 6.25), or 27.5 mg. of albumin (N × 6.25).

The tyrosine equivalents of the plasma proteins of different species of animals are probably different and should be determined when required. To do this it is only necessary to make parallel determinations on the same sample of plasma by the new colorimetric method and by the Kjeldahl method.

In the method which follows the fibrin and the albumin are determined directly, while the globulin is determined by the difference between the total serum proteins and the albumin. All the determinations can be made simultaneously and finished in 1 hour.

Determination of the Fibrin.—To 1 cc. of the plasma (from blood containing 0.2 to 0.6 per cent potassium oxalate) add 28 cc. of 0.8 per cent NaCl solution and 1 cc. of 2.5 per cent $CaCl_2$ solution. Mix and allow to stand undisturbed for 20 minutes. Break up the jelly by shaking slightly and transfer it to a dry filter. While filtering insert into the jelly a slender glass rod with a pointed end

[1] We used Pfanstiehl tyrosine prepared by the Special Chemicals Company, Highland Park, Ill.

and whirl gently. All the fibrin will stick to the rod.[2] Slip the fibrin off the rod, and press it between dry filter paper to remove as completely as possible the adhering liquid. Transfer it to a 15 cc. centrifuge tube, add 4 cc. of 1 per cent sodium hydroxide. Place the tube in a boiling water bath and stir with a slender glass rod until the fibrin lump has completely disintegrated. The fibrin has now dissolved, leaving the calcium oxalate in suspension. Add 10 cc. of water, mix, and centrifuge. Transfer the supernatant liquid to a 25 cc. volumetic flask or graduated tube. Cool under the tap. Add 1 cc. of 5 per cent H_2SO_4, 0.5 cc. of phenol reagent, and dilute to about 20 cc. Add 3 cc. of 20 per cent Na_2CO_3 solution. Shake. Add 1 drop of ether to dissipate the foam, make up to volume, and mix. The standard is prepared as follows: Measure 1 cc. of the standard tyrosine solution into a 25 cc. volumetric flask or graduated tube, add 0.5 cc. of phenol reagent, dilute to about 20 cc., and finally add 3 cc. of 20 per cent Na_2CO_3 solution. Make up to volume and mix. The standard should, of course, be prepared at the same time as the unknown. Let stand for 15 minutes before making the color comparison.

Calculation.—If the standard is set at 20 and the reading of the unknown is R, then the amount of the apparent tyrosine determined is $\frac{20}{R} \times 0.2$ mg. Since 1 mg. of tyrosine = 16.4 mg. of fibrin, the amount of fibrin in 1 cc. of plasma is $\frac{20}{R} \times 0.2 \times 16.4$ mg. or the percentage of fibrin $= \frac{20}{R} \times 0.328$.

Determination of Albumin.—To 1 cc. of plasma add 9 cc. of $\frac{5}{9}$ saturated ammonium sulfate solution[3] or 9 cc. of saturated magnesium sulfate solution and 0.3 gm. of anhydrous magnesium sulfate. Mix and allow to stand for 30 minutes. Filter. Measure 1 cc. of the filtrate into a 15 cc. centrifuge tube. Add about

[2] If any fibrin fails to stick to the rod it should be picked up with the tip of the rod. It requires no great skill to do this successfully. If the amount of the fibrin in the plasma is very high, say 0.8 per cent or more, the fibrin jelly will not shrink readily. In such a case it is necessary to use less of the plasma for the determination.

[3] This is made by diluting 555 cc. of saturated ammonium sulfate to 1 liter.

12 cc. of H_2O, 1 cc. of 10 per cent sodium tungstate solution, and then 1 cc. of $\frac{2}{3}$ N sulfuric acid. The amount of the tungstate solution and the sulfuric acid need not be exactly measured, but care must be taken to see that the volume of the acid used is at least equal to that of the tungstate solution. Stir thoroughly with a slender glass rod and centrifuge. Carefully decant off the supernatant liquid as completely as possible. (The volume of the wet precipitate usually amounts to about 0.5 cc. If it is much smaller than 0.5 cc., indicating low albumin, measure another cc. of albumin filtrate into the same tube, dilute with water and proceed as before.) Add to the precipitate in the centrifuge tube 1 cc. of sodium tungstate solution. Stir until the precipitate has dissolved, dilute with 13 cc. of H_2O, and add 1 cc. of H_2SO_4. Stir again, centrifuge, and decant off the supernatant liquid. This second precipitation is intended to remove the calcium and ammonium or magnesium so nearly completely that they cannot possibly interfere with the subsequent color reaction, although experience has shown that a single precipitation usually suffices. Add to the precipitate in the tube 10 cc. of H_2O and 1 or 2 drops (but no more) of 20 per cent Na_2CO_3. Stir until the precipitate has dissolved. Transfer the resulting solution to a 25 cc. volumetric flask or graduated tube. Rinse the centrifuge tube twice with 3 cc. of H_2O. Add 0.5 cc. of phenol reagent and 3 cc. of 20 per cent Na_2CO_3 solution. Shake. Add 1 or 2 drops of ether to dissipate the foam. Make up to volume and mix. Prepare a standard as in the fibrin determination and read the color after 15 minutes.

Determination of Albumin and Globulin.—Measure 2 cc. of the filtrate in the fibrin determination into a 15 cc. centrifuge tube and proceed exactly as in the determination of albumin.

Calculation.—In the calculation it is to be noted that the solution used for the albumin determination is plasma diluted 1 to 10, while that used for the determination of albumin and globulin is plasma diluted 1 to 30. If 1 cc. of the albumin solution is used for the former determination and 2 cc. of the serum solution are used for the latter determination and the colorimeter readings are R_a and R_t respectively, the standard being set at 20, then the total apparent tyrosine in 1 cc. of serum $= 15 \times \frac{20}{R_t} \times 0.2$ mg.,

the apparent tyrosine of albumin in 1 cc. of serum = $10 \times \frac{20}{R_a} \times 0.2$ mg., and the apparent tyrosine of globulin in 1 cc. of serum is

$$(15 \times \frac{20}{R_t} \times 0.2) - (10 \times \frac{20}{R_a} \times 0.2) = \frac{60}{R_t} - \frac{40}{R_a} \text{ mg.}$$

Since 1 mg. of tyrosine = 25.2 mg. of globulin = 27.5 mg. of albumin,

$$\therefore \text{ per cent of globulin} = \frac{\left(\frac{60}{R_t} - \frac{40}{R_a}\right) \times 25.2}{1{,}000} \times 100$$

$$= \left(\frac{6}{R_t} - \frac{4}{R_a}\right) \times 25.2$$

$$\text{per cent of albumin} = \frac{\left(10 \times \frac{20}{R_a} \times 0.2\right) \times 27.5}{1{,}000} \times 100 = \frac{20}{R_a} \times 5.44$$

EXPERIMENTAL.

Determination of the Tyrosine Equivalent of the Plasma Proteins.

Fibrin.—The fibrin in about 5 cc. of human plasma was isolated as described. It was placed in a 25 cc. volumetric flask and 20 cc. of 1 per cent NaOH were added. The flask was placed in boiling water with frequent shaking. When the fibrin lump had completely disintegrated the flask was cooled under the tap and the solution was made up to volume with 5 per cent H_2SO_4 and mixed. It was filtered to remove the calcium oxalate. 5 cc. of the filtrate were taken for the tyrosine determination as described above. The nitrogen was determined in 2 or 3 cc. of the filtrate by the micro Kjeldahl method (5). It was found in two experiments representing two samples of plasma that 1 mg. of tyrosine = 16.8 and 16.0 mg., respectively, averaging 16.4 mg. of fibrin (N × 6.25).

Albumin.—The globulin together with fibrin was precipitated from the plasma using magnesium sulfate as described. 1 cc. of the filtrate was taken for the tyrosine determination. 5 cc. of the filtrate were measured into a 10 cc. volumetric flask, diluted to volume, and mixed. 1 cc. of this diluted solution was measured into a Pyrex test-tube, 2 cc. of digesting mixture were added, and

the digestion was carried out in the usual way (5). 25 cc. of H_2O were added and the solution was cooled and transferred to a very large test-tube or small flask. 25 cc. of 10 per cent NaOH were then added and the ammonia was aspirated and determined by Nesslerization in the usual manner. The ammonia in the reagents and the non-protein nitrogen of the plasma were deter-

TABLE I.

*Plasma Proteins in Normal and Pathological Blood.**

No.	Diagnosis.	Fibrin.	Total serum protein.	Albumin.	Globulin.
		per cent	*per cent*	*per cent*	*per cent*
1	Normal		6.50	4.55	1.95
2	"		6.55	4.80	1.75
3	"		7.51	5.28	2.23
4	"	0.222	6.62	4.75	1.87
5	"		7.54	4.89	2.65
6	Syphilis		8.04	4.63	3.41
7	"		9.37	4.95	4.42
8	Kala-azar		9.59	3.83	5.76
9	"		10.54	3.48	7.06
10	"		6.78	2.90	3.88
11	"		7.37	2.94	4.43
12	"	0.271	6.99	1.82	5.17
13	"	0.306	7.82	2.35	5.47
14	" treated	0.290	7.40	4.05	3.35
15	"	0.292	7.79	2.62	5.17
16	"		7.98	1.94	6.04
17	Streptococcus septicemia		5.56	2.76	2.80
18	Amebic dysentery		4.24	2.90	1.34
19	Relapsing fever	0.485	5.57	2.58	2.99
20	Nephritis with edema	0.630	3.07	0.53	2.54
21	Anemia		7.50	5.00	2.50

* Some of the figures in this table have been reported elsewhere (1).

mined and corrections were made accordingly. In five experiments representing five specimens of plasma it was found that 1 mg. of tyrosine = 27.6, 27.4, 27.5, 27.3, and 27.8 mg., respectively; averaging 27.5 mg. of albumin (N × 6.25).

Globulin.—1 cc. of the serum filtrate of the same plasma used for the albumin determination was measured into a Pyrex test-tube and the nitrogen was determined exactly as in the preceding ex-

periment. In another 2 cc. of the filtrate the tyrosine was determined. It was found by the method of calculation indicated above that 1 mg. of tyrosine = 24.9, 25.8, 24.7, 24.9, and 25.6 mg., respectively; averaging 25.2 mg. of globulin (N × 6.25).

It would appear from the values of tyrosine equivalents given above that the fibrin contains much more tyrosine than the albumin or the globulin. But this is not the case. We have observed that the chromogenic value of the albumin or the globulin is greatly increased by treating with NaOH for a few minutes or with Na_2CO_3 on long standing. The apparently high tyrosine content of the fibrin is no doubt due to the action of NaOH used to bring it into solution.

In Table I are shown some of the results obtained with the new method. It is not the purpose of the present paper to discuss the significance of the findings in those pathological cases reported here, but the deviation from normal is sometimes so marked that the clinical value of the determination of plasma proteins is apparent.

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